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Genschow et al.
In Vitro & Molecular Toxicology
13(1):51-66
2000

Scholz et al.
Cells Tissues Organs
165(3-4):203-211
1999

Spielmann et al.
In Vitro Toxicology: Journal of Molecular and Cellular Toxicology
10(1):119-127
1997

Mummery et al.
Reproductive Toxicology
7(Suppl. 1):145-154
1993

Craig et al.
Biomarkers
1(2):123-135
1996

Webster, W.S.
Congenital Anom.
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1989

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ECVAM's in-house prevalidation/validation studies in the areas of haematotoxicity, reproductive toxicity, metabolism-mediated toxicity and epithelial barrier function

Pilar Prieto*

European Commission, Institute for Health and Consumer Protection, Joint Research Centre, ECVAM, 21020 Ispra, Varese, Italy

Abstract

The European Centre for the Validation of Alternative Methods (ECVAM) facilitates, co-ordinates and participates in validation activities at the European Union level. Various experimental studies, e.g. in the areas of haematotoxicity, reproductive toxicity, nephrotoxicity and epithelial barrier function, and metabolism-mediated toxicity, are underway in ECVAM's laboratories. ECVAM itself is currently involved in the prevalidation/validation of two assays, the colony-forming unit granulocyte/macrophage (CFU-GM) assays for predicting acute neutropenia and the embryonic stem cell test for predicting embryotoxicity. In the areas of metabolism-mediated toxicity and nephrotoxicity and epithelial barrier function, several assays are in the course of development. In many cases, the recommendations of various ECVAM workshops are being followed.

Keywords: ECVAM; In vitro toxicology; Prevalidation/validation studies

1. Introduction

The European Centre for the Validation of Alternative Methods (ECVAM) was established by the European Commission to co-ordinate the validation of alternative test methods at the European Union level; to act as a focal point for the exchange of information on the development of alternative test methods; to establish, maintain and manage a database on alternative procedures; and to promote dialogue among legislators, industries, biomedical scientists, consumer organ-

*Tel.: +39-0332-785534; fax: +39-0332-785336.
E-mail address: maria.pricto – pilar@jrc.it (P. Prieto)

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isations and animal welfare groups, with a view to the development, validation and international recognition of alternative test methods.

ECVAM seeks to promote the scientific and regulatory acceptance of alternative methods which are of importance to the biosciences and which can *reduce, refine or replace* the use of laboratory animals (Russell and Burch, 1959).

One of the first priorities of ECVAM is to ensure that it is well informed about the state-of-the-art of non-animal test development and validation, and the potential for the possible incorporation of alternative tests into regulatory procedures. To achieve this goal, ECVAM organises workshops on specific topics to review the current status of various types of in vitro tests and their potential uses and make recommendations about the best way forward (Anon., 1994). More than 35 workshops have been held so far, covering several areas such as hepatotoxicity (Blaauboer et al., 1994), phototoxicity (Spielmann et al., 1994), neurotoxicity (Atterwill et al., 1994), immunobiologicals (Hendriksen et al., 1994), validation of toxicity tests procedures (Balls et al., 1995), skin corrosivity testing (Botham et al., 1995), nephrotoxicity (Hawksworth et al., 1995), reproductive toxicity (Brown et al., 1995), percutaneous absorption (Howes et al., 1996), haematotoxicity (Gribaldo et al., 1996), biokinetics (Blaauboer et al., 1996), acute toxicity and the classification and labelling of chemicals (Siebert et al., 1996), medical devices (Svendsen et al., 1996), respiratory toxicity (Lambré et al., 1996), skin sensitisation testing (de Silva et al., 1996), tissue slices for pharmacotoxicology studies (Bach et al., 1996), pharmacokinetics (Leahy et al., 1997), monoclonal antibody production (Marx et al., 1997), expert systems for predicting toxicity (Dearden et al., 1997), databases on alternative methods (Janusch et al., 1997), genetically engineered cell lines (Wiebel et al., 1997), transgenic animals in the European Union (Mepham et al., 1998), and potency testing of vaccines (Hendriksen et al., 1998). The reports and recommendations of these workshops are published in *ATLA*.

Nine ECVAM task forces have been established to focus on more specific issues and on ways of implementing the recommendations of workshops in the planning of prevalidation/validation studies and of seeking acceptance of the outcome of successful validation studies: they are concerned with prevalidation, biostatistics, hepatocytes/metabolism, hormones, nephrotoxicity, neurotoxicity, developmental toxicity, skin irritation, and integrated systems. Four ECVAM task force reports have been published so far (Curren et al., 1995; Holzhütter et al., 1996; Morin et al., 1997; Botham et al., 1998).

Following the recommendations of some of the ECVAM workshops (Spielmann et al., 1994; Botham et al., 1995; Garthoff et al., 1995) and task forces (Botham et al., 1998), ECVAM has co-ordinated and funded several prevalidation and validation studies in recent years: e.g. the EU/COLIPA validation study on in vitro tests for phototoxicity (the main test evaluated in this study is the 3T3-neutral red uptake phototoxicity test (see Spielmann et al., 1995a,b, 1998), and on ECVAM prevalidation and validation study on in vitro tests for skin corrosivity (two of four tests, EPISKIN™ and the rat skin transcutaneous electrical resistance assay, were judged to have been scientifically validated). Several international studies on in vitro methods for vaccine potency and safety testing have been initiated.

Furthermore, experimental studies in collaboration with research groups in the EU Member States are undertaken in ECVAM's own laboratories, the main studies being in the areas of embryotoxicity, haematotoxicity, metabolism-mediated toxicity, and epithelial barrier function and nephrotoxicity. The laboratory work is concerned with the evaluation, prevalidation and validation of new in vitro tests.

2. Area descriptions

2.1. Haematotoxicity

Following the recommendations of the ECVAM workshop 14 (Gribaldo et al., 1996), a prevalidation/validation study is in progress on a colony-forming unit granulocyte/macrophage (CFU-GM) assay for predicting acute neutropenia. The test has been selected on the basis of its

potential usefulness for regulatory testing purposes, similarities in the protocols which had been derived independently by several laboratories, and evidence of success in predicting toxicity (Pessina et al., 1989; Du et al., 1992; Parchment et al., 1993, 1998; Volpe et al., 1995; Heuvel et al., 1997). The study is being conducted in two phases. The first phase involves prevalidation and database development, in which protocols for procedures using the murine bone marrow cells and human umbilical cord blood cells have been optimised. Two standard operating procedures (SOPs) have been generated. A comprehensive database (HEMATOX) of in vivo and in vitro data on the hematotoxicity of compounds, for use in the prevalidation/validation of the CFU-GM assay, has been established and can be consulted on the Internet at the URL address: http://www2.vito.be/dbtoep/hematox/hematoxframe.htm

The second phase is the validation phase, which involves a blind trial with coded chemicals to determine the value of the in vitro assay for predicting for acute neutropenia in vivo (in mice and humans).

The protocols and results of the prevalidation and validation phases will be available when the management team's report on the outcome of the validation study is published. Meanwhile research is in progress on tests for effects on other haematopoietic lineage.

2.2. Reproductive toxicity

Developmental toxicity is an important and highly complex aspect of reproductive toxicology. The prediction of potential damage to the developing embryo and foetus currently involves animal procedures which are expensive and unpleasant, since large number of pregnant animals have to be killed. Therefore, the search for relevant and reliable replacement alternatives is a high priority.

Following the recommendations of ECVAM workshop 12 (Brown et al., 1995), a study was set up on the prevalidation/validation of three embryotoxicity tests: the micromass test (Umansky, 1996; Flint, 1993), the post-implantation rat whole-embryo culture assay (Bechter et al., 1992), and a test employing embryonic stem cell lines (Spielmann et al., 1997). ECVAM itself is participating in the prevalidation/validation of the embryonic stem cell test. The aim of this assay is to assess the specific embryotoxic potentials of chemicals by comparing general cytotoxicity exerted to 3T3 fibroblasts with toxic effects observed in embryonic stem cells (D3) and effects on the differentiation of these stem cells into cardiac myoblasts.

By using a specific culture system [the hanging drop method according to Wobus et al. (1991)] and a supplemented DMEM culture medium, the cells are induced to form embryoid bodies which differentiate into cardiac muscle cells (Heuer et al., 1994; Spielmann et al., 1995a,b). The teratogenic and embryotoxic effects of compounds are evaluated by means of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Mosmann 1983), the percentage of embryonic bodies beating, mRNA expression of α and β myosin heavy chains, and quantification of specific gene expression (cardiac specific promoter-α actin, and a reporter gene which codes for a green fluorescent protein; Bremer et al., 1996).

During the prevalidation phase, a SOP describing the technical details of the embryonic stem cell test, positive and negative controls, prediction models, and chemicals to be assayed, was prepared by the lead laboratory. The inter-laboratory transferability of the in vitro test was determined by transferring the SOP from the lead laboratory to the refinement laboratory (Curren et al., 1995). The statistical analysis of data obtained during this phase showed reproducible results between the lead laboratory and the refinement laboratory (results not shown). In addition, the prediction models developed in the lead laboratory provided a correct classification of the embryotoxic potentials of the chemicals selected (results not shown). The test is now ready to undergo formal validation in a blind trial according to the criteria set by ECVAM (Balls et al., 1995).

The test protocols and the data generated during this prevalidation/validation study will be available when the report on the outcome of the study is published by the management team.

2.3. Nephrotoxicity and epithelial barrier function

The body has epithelial barrier systems of various kinds. The major functions of epithelia are the regulation of permeability, transport, endocytosis and exocytosis. At present, the main focus at ECVAM is the renal tubular epithelium. New screening procedures which can rapidly and reliably detect renal epithelial toxicity are in the course of development, and are based on the use of several renal epithelial cell lines (MDCK dog distal tubule cell line, LLC-PK1 pig proximal tubule cell line, and OK opossum proximal tubule cell line).

Following the recommendations of ECVAM workshop 10 (Hawksworth et al., 1995) barrier function tests are also being developed. The trans-epithelial electrical resistance measurement technique is an easy and relatively inexpensive electrophysiological method which, together with the assessment of the leakage of fluorescein isothiocyanate-inulin through cell monolayers grown on microporous supports, is a good indicator of the barrier functions of all epithelial systems (Powell 1981; Rabito 1986; Steinmassl et al., 1995).

A task force on nephrotoxicity (Morin et al., 1997) has been established to follow up these recommendations in the workshop report, and is now producing a strategy for ECVAM in relation to the harmonisation of technologies to obtain well-characterised human renal epithelial cells for nephrotoxicty studies.

ECVAM is also participating in the prevalidation/validation of the fluorescein leakage in vitro eye irritancy test. The aim of the assay is to predict the eye irritation potential of tests materials by looking at the integrity of the tight cell–cell junctions of MDCK cells (Tchao 1988; Atkinson et al., 1995).

2.4. Metabolism-mediated toxicity

Another area of interest is metabolism-mediated toxicity, an important aspect of almost all in vitro toxicology studies and toxicity tests. There is an urgent need for screening procedures incorporating the main enzyme systems involved.

Following the recommendations of ECVAM workshop 26 (Wiebel et al., 1997), in vitro screening systems capable of predicting metabolism-mediated toxicity are being developed in order to provide information that can be used in human risk assessment. Various genetically engineered mammalian cell lines transfected with human cytochrome P450 cDNAs are being used. After the full characterisation of three mammalian host cell lines (AHH-1 lymphoblastoid cell line, HIH-3T3 mouse embryo cell line, and V79 Chinese hamster lung cell line), scientifically acceptable screening metabolism models will be further developed and validated to identify problem test compounds with regard to metabolism-mediated toxicity, and incorporating other endpoints such as metabolic lability inhibition and human drug polymorphism.

3. Discussion

Since ECVAM's establishment in 1991 by the European Commission, ECVAM's activities are based on criteria established with its independent Scientific Advisory Committee and in consultation with other Commission services, especially DG XI. Due to the range and quality of its activities, ECVAM is now known as one of the world's most significant 'Three Rs' organisations.

The principles of validation and regulatory acceptance have been clearly defined and harmonised at the international level. In addition, the quality and efficiency of the validation process have been improved. A number of studies have been completed, and three methods (the 3T3-neutral red uptake phototoxicity test, and the EPISKIN™ and the rat skin transcutaneous electrical resistance assay for corrosivity) have been validated according to the current ECVAM criteria. Draft test guidelines have been submitted to the Commission and the OECD.

Prevalidation/validation studies are now beginning on in vitro methods for the blood brain barrier, skin irritation testing, and a reference standard approach for replacing the Draize eye test.

A database on alternative methods and related

information has been created by ECVAM. At present factual (real data and not only bibliographic references) and evaluated information in priority topics is provided. Data collection is now started for other topics. A protocol database is already running, at ECVAM, as well as a database on validation studies and a database for the ECVAM workshop reports and task forces.

The ECVAM laboratories provide support of various kinds to prevalidation and/or validation studies in areas such as embryotoxicity, haematotoxicity, nephrotoxicity and epithelial barrier function, and metabolism-mediated toxicity. The prevalidation phase of the prevalidation/validation studies under way in the areas of haematotoxicity and embryotoxicity have successfully finished and the validation phase has already started.

References

Anon. ECVAM news and views. ATLA 1994;22(1):7–11.

Atterwill CK, Bruinink A, Drejer J et al. In vitro neurotoxicity testing. The report and recommendations of ECVAM workshop 13. ATLA 1994;22(5):350–362.

Atkinson K, Fentem J, Balls M. A survey of alternatives to the Draize eye irritation test and of their current state of validation. Report prepared for DG XI/C.6 of the European Commission. Contract 89/X1/A2-6 1995. Nottingham: FRAME, 1995.

Bach PH, Vickers AEM, Fischer R et al. The use of tissue slices for pharmacotoxicology studies. The report and recommendations of ECVAM workshop 20. ATLA 1996; 24(6):893–923.

Balls M, Blaauboer B, Fentem JH et al. Practical aspects of the validation of toxicity test procedures. The report and recommendations of ECVAM workshop 5. ATLA 1995; 23(1):129–147.

Bechter R, Terlouw GDC, Tsuchiya M, Tsuchiya T, Kistler A. Teratogenicity of arotinoids (retinoids) in the rat-whole embryo culture. Arch Toxicol 1992;66:193–197.

Blaauboer BJ, Boobis AR, Castell JV et al. The practical application of hepatocytes cultures in routine testing. The report and recommendations of the ECVAM workshop 1. ATLA 1994;22(4):231–241.

Blaauboer BJ, Bayliss MK, Castell JV et al. The use of biokinetics and in vitro methods in toxicological risk evaluation. The report and recommendations of ECVAM workshop 15. ATLA 1996;24(4):473–497.

Botham PA, Chamberlain M, Barratt MD et al. A prevalidation study on in vitro skin corrosivity testing. The report and recommendations of the ECVAM workshop 6. ATLA 1995;23(2):219–255.

Botham PA, Earl LK, Fentem JH, Roguet R, van de Sandt JJM. Alternative methods for skin irritation testing: the current status. ECVAM Skin Irritation Task Force report 1. ATLA 1998;26(2):195–211.

Bremer S, Genart C, Bigot K, Clerici L, Spielmann H, Balls M. Immunofluorescence studies of differentiating embryonic stem cells [special issue]. Alternatives Lab Anim 1996; 24:237.

Brown NA, Spielmann H, Bechter R et al. Screening chemicals for reproductive toxicity: the current alternatives. The report and recommendations of the ECVAM workshop 12. ATLA 1995;23(6):868–882.

Curren RD, Southee JA, Spielmann H, Liebsch M, Fentem JH, Balls M. The role of prevalidation in the development, validation and acceptance of alternative methods. ECVAM Prevalidation Task Force report 1. ATLA 1995;23(2): 211–217.

de Silva O, Basketter DA, Barratt MD et al. Alternative methods for skin sensitisation testing. The report and recommendations of ECVAM workshop 19. ATLA 1996; 24(5):683–705.

Dearden JC, Barratt MD, Benigni R et al. The development and validation of expert systems for predicting toxicity. The report and recommendations of ECVAM/ECB workshop (ECVAM workshop 24). ATLA 1997;25(3):223–252.

Du DL, Volpe DA, Grieshaber CK, Murphy MJ. In vitro toxicity of 3′-azido-3′-deoxythymidine (AZT), Carbovir, and 2′,3′-dideoxythymidine (d4T) to human and murin hematopoietic progenitor cells. Br J Haematol 1992;80: 437–445.

Flint OP. In vitro tests for teratogens: desirable endpoints, test batteries and current status of the micromass teratogen test. Reprod Toxicol 1993;7(1):103–111.

Gribaldo L, Bueren J, Deldar A et al. The use of in vitro systems for evaluating haematotoxicity. The report and recommendations of the ECVAM workshop 14. ATLA 1996;24(2):211–231.

Hawksworth GM, Bach PH, Nagelkerke JF et al. Nephrotoxicity testing in vitro. The report and recommendations of the ECVAM workshop 10. ATLA 1995;23(5):713–727.

Hendriksen CFM, Garthoff H, Aggerbeck H et al. Alternatives to animal testing in the quality control of immunobiologicals: current status and future prospects. The report and recommendations of ECVAM workshop 4. ATLA 1994;22(6):420–434.

Hendriksen C, Spieser J-M, Akkermans A et al. Validation of alternative methods for the potency testing of vaccines. The report and recommendations of ECVAM workshop 31. ATLA 1998;26(6):747–761.

Heuer J, Graeber IM, Pohl I, Spielmann H. Culture system for the differentiation of murine embryonic stem cells — a new approach to in vitro testing for embryotoxicity and for developmental immunotoxicology. In: Fracchia GN, editor. European medicines research. Amsterdam: IOS press, 1994:134–145.

Heuvel van den RL, Schoeters G, Leppens H. Haematotoxicity testing in-vitro using human cord blood haematopoietic cells. Toxicol Vitro 1997;11:689–693.

Holzhütter H-G, Archer G, Dami N, Lovell DP, Saltelli A, Sjöström M. Recommendation for the application of biostatistical methods during the development and validation of alternative toxicological methods. ECVAM Biostatistics Task Force report 1. ATLA 1996;24(4):511–530.

Howes D, Guy R, Hadgraft J et al. Methods for assessing percutaneous absorption. The report and recommendations of the ECVAM workshop 13. ATLA 1996;24(1):81–106.

Janusch A, van der Kamp MDO, Bottrill K et al. Current status and future developments of databases on alternative methods. The report and recommendations of the ECVAM workshop 25. ATLA 1997;25(4):411–422.

Lambré CR, Aufderheide M, Bolton RE et al. In vitro tests for respiratory toxicity. The report and recommendations of the ECVAM workshop 18. ATLA 1996;24(5):671–681.

Leahy DE, Duncan R, Ahr HJ et al. Pharmacokinetics in early drug research. The report and recommendations of the ECVAM workshop 22. ATLA 1997;25(1):17–31.

Marx U, Embleton MJ, Fischer R et al. Monoclonal antibody production. The report and recommendations of the ECVAM workshop 23. ATLA 1997;25(2):121–137.

Mepham TB, Combes RD, Balls M et al. The use of transgenic animals in the European Union. The report and recommendations of the ECVAM workshop 28. ATLA 1998;26:21–43.

Morin J-P, De Broe ME, Pfaller W, Schmuck G. Nephrotoxicity testing in vitro: the current situation. ECVAM Nephrotoxicity Task Force report 1. ATLA 1997;25(5):497–503.

Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 1983;65:55–63.

Parchment RE, Huang M, Sachs L. Roles for in vitro myelotoxicity tests in preclinical drug development and clinical trial planning. Toxicol Pathol 1993;21:241–250.

Parchment RE, Gordon M, Grieshaber CK, Sessa C, Volpe D, Chielmini M. Predicting hematological toxicity (myelosuppression) of cytotoxic drug therapy from in vitro tests. Ann Oncol 1998;9(4):357–364.

Pessina A, Neri MG, Muschiato A, Mineo E, Cocuzza G. Effect of fluoroquinolones on the in vitro proliferation of myeloid precursor cells. J Antimicrob Chemother 1989; 24:203–208.

Powell DW. Barrier function of epithelia. Am J Physiol 1981;241:G275–G288.

Rabito CA. Occluding junctions in a renal cell line (LLC-PK1) with characteristics of proximal tubular cells. Am J Physiol 1986;250:F734–F743.

Russell WMS, Burch RL. The principles of humane experimental technique. London: Methuen, 1959.

Siebert H, Balls M, Fentem JH et al. Acute toxicity testing in vitro and the classification and labelling of chemicals. The report and recommendations of the ECVAM workshop 16. ATLA 1996;24(4):499–510.

Spielmann H, Lowell WW, Hölzle E et al. In vitro phototoxicity testing. The report and recommendations of ECVAM workshop 2. ATLA 1994;22(5):314–348.

Spielmann H, Pohl I, Döring B, Liebsch M, Moldenhauer F. In vitro embryotoxicity assay using two permanent cell lines: mouse embryonic stem cells and 3T3 fibroblasts. Abstracts of the 23rd ETS Conference 1995, Dublin. Teratology 1995a;51(6):31A–32.

Spielmann H, Liebsch M, Pape WJW et al. The EEC/COLIPA in vitro photoirritancy program: results of the first stage of validation. In: Elsner P, Maibach HI, editors. Irritant dermatitis: new clinical and experimental aspects, 1995b:256–264.

Spielmann H, Pohl I, Döring B, Liebsch M, Moldenhauer F. The embryonic stem cell test (EST), an in vitro embryotoxicity test using two permanent mouse cell lines: 3T3 fibroblasts and embryonic stem cells. Toxicol Vitro 1997;10(1): 119–127.

Spielmann H, Balls M, Dupuis J et al. EU/COLIPA 'In vitro phototoxicity' validation study, results of phase II (blind trial), part 1: the 3T3 NRU phototoxicity test. Toxicol Vitro 1998;12:305–327.

Steinmassl D, Pfaller W, Gstraunthaler G, Hoffman W. LLC-PK1 cells as a model for in vitro assessment of proximal tubular nephrotoxicity. In Vitro Cell Dev Biol 1995;31: 94–106.

Svendsen O, Garthoff B, Spielmann H et al. Alternatives to animal testing of medical devices. The report and recommendations of the ECVAM workshop 17. ATLA 1996; 24(5):659–669.

Tchao R. Trans-epithelial permeability of fluorescein in vitro as an assay to determine eye irritation. In: Goldberg AM, editor. Alternative methods in toxicology, progress in in vitro toxicology, 6. New York: Mary Ann Liebert, Inc, 1988:271–283.

Umansky R. The effect of cell population density on the developmental fate of reaggregating mouse limb bud mesenchyme. Devel Biol 1996;13:31–56.

Volpe DA, Cole K, Sandeen A, Kohn EC. In vitro and in vivo myelotoxicity of CAI to human and murine hematopoietic progenitor cells. Am J Hematol 1995;50:277–282.

Wiebel FJ, Andersson TB, Casciano DA et al. Genetically engineered cell lines: characterisation and applications in toxicity testing. The report and recommendations of the ECVAM workshop 26. ATLA 1997;25(6):625–639.

Wobus AM, Wallukat G, Hescheler J. Pluripotent mouse embryonic stem cells are able to differentiate into cardiomyocites expressing chronotropic responses to adrenergic and cholinergic agents and Ca^{2+} channel blockers. Differentiation 1991;48:173–182.